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(71)(72) Applicant and Inventor: YOUNG, Alexander, T. [US/US]; Flagship Wharf 719, 197 at the Navy Yard, Eighth Street, Charlestown, MA 02129 (US).

(74) Agent: CLARK, Paul, T.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US).

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(54) Title: GENE THERAPY USING TARGETED VIRAL VECTORS

(57) Abstract

A general method for delivering genes to specific target cells *in vivo* is described. Enveloped viruses are genetically engineered to infect specific target cells by replacing the cell surface receptor recognition domain of viral envelope proteins with ligands that direct the binding and fusion of these viruses to specific cell surface molecules.

GENE THERAPY USING TARGETED VIRAL VECTORS

Background of the Invention

The invention relates to gene therapy methods.

5   Gene therapy is an approach to treating a broad range of diseases by delivering therapeutic genes directly into the human body. Diseases that can potentially be cured by gene therapy include 1) diseases associated with the aging population such as cancer,
10 heart disease, Alzheimer's disease, high blood pressure, atherosclerosis and arthritis; 2) viral infectious diseases such as acquired immune deficiency syndrome (AIDS) and herpes; and 3) inherited diseases such as diabetes, hemophilia, cystic fibrosis, and muscular
15 dystrophy.

Current methods of delivery of new genetic information into cells *in vitro* include cell fusion, chromosome-mediated insertion, microcell-mediated gene transfer, liposome DNA carriers, spheroplast fusion, DNA-
20 mediated gene transfer, microinjection, infection with recombinant RNA viruses, and infection with recombinant DNA viruses (Martin, J.C., 1984, *Mol. Cell Biochem.* 59:3-10). These techniques are not generally applicable, however, for use in animals or humans because of low
25 efficiency, instability of introduced genes, introduction of extraneous or undesirable genetic information, and lack of target specificity.

In one particular example, a favored approach for human gene therapy involves the transplantation of
30 genetically-altered cells into patients (Rosenberg, et al., 1988, *New Eng J Medicine* 323:570-578). This approach requires the surgical removal of cells from each patient to isolate target cells from nontarget cells. Genes are introduced into these cells via viral vectors
35 or other means, followed by transplantation of the

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genetically-altered cells back into the patient.
Although this approach is useful for purposes such as
enzyme replacement therapy (for example, for
transplantation into a patient of cells that secrete a
5   hormone that diseased cells can no longer secrete),
transplantation strategies are less likely to be suitable
for treating diseases such as cystic fibrosis or cancer,
where the diseased cells themselves must be corrected.
Other problems commonly encountered with this approach
10 include technical problems, including inefficient
transduction of stem cells, low expression of the
transgene, and growth of cells in tissue culture which
may select for cells that are predisposed to cancer.
Finally, inappropriate expression of transplanted genes
15 in nontarget cells may actually be harmful to patients.

An alternative approach to gene therapy involves
the direct delivery of genes to target tissue *in situ*.
Two methods for *in situ* delivery of genes have been
developed: biolistic transfer and double balloon
20 catheterization. Biolistic transfer of genes involves
shooting DNA-coated platinum or gold microprojectiles
directly into target tissue. Biolistic transfer has been
successful in the transient expression of genes in the
ear, skin and surgically-exposed liver of live mice
25 (Johnston, S.A., 1990, *Nature* 346:776-777; Williams,
R.S., et al., 1991, *Proc Natl Acad Sci USA* 88:2726-2730).
Double balloon catheterization transduces genes into
cells within a defined arterial wall segment. In this
approach a double balloon catheter is inserted into an
30 artery until the end of the catheter is located within
the target area. Inflation of two balloons at the end of
the catheter creates an enclosed space into which
retrovirus or DNA-loaded liposomes are infused. This
method has been successful in the transient expression of
35 β-galactosidase genes within a defined segment of the

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ileofemoral artery of pigs (Nabel E.G., et al., 1990,
Science 249:1285-1288). Both biolistic transfer and
double balloon catheterization however, although locally
specific, may be nonspecific in the individual cells that
5 they transduce within the target area, creating a problem
of inappropriate gene regulation if the transgene is
expressed in nontarget cells. Moreover, neither
biolistic transfer nor double balloon catheterization
have been shown to be effective for the treatment of
10 tissue occupying large volumes such as lungs, muscles,
tumors, or cells of the systemic circulation since the
majority of the cells would be inaccessible for *in situ*
gene transfer.

A third approach to gene therapy is the delivery
15 of genes to cells *in vivo*. This approach involves the
introduction of viral vectors directly into patients by
injection, spray or other means. Different species of
viruses are engineered to deliver genes to the cells that
the viruses normally infect. Adenovirus, for example,
20 which normally infects lung cells, has been developed as
a vector to target genes to lung cells (Rosenfield, et
al., 1992, *Cell* 68 143-155). Most viral vectors,
however, are single purpose vectors since they can only
deliver genes to certain cells. Because the target cell
25 specificity of viral vectors is restricted to the normal
tropisms of the viruses, viral vectors are generally
limited in that they either infect too broad a range of
cell types, or they do not infect certain types of cells
at all.

30   Liposomes have been designed to deliver genes or
drugs to specific target cells *in vivo*. By chemically
conjugating antibodies or ligands to liposomes, liposomes
have been targeted to specific cells. With this method,
antisense *env* RNA has been delivered to human
35 immunodeficiency virus (HIV)-infected lymphocytes using

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anti-CD3-conjugated liposomes (Renneisen, K., et al.,
1990, *J Biol Chem* 265:16337-16342); chloramphenicol
transacetylase (CAT) genes have been delivered to H2K^k
positive lymphomas in H2K^k-negative nude mice using anti-
5   H2K^k-conjugated liposomes (Wang, C. et al., 1987, *Proc
Natl Acad Sci USA* 84:7851-7855); and xanthine guanine
phosphoribosyltransferase (XGPRT) genes have been
delivered to immunoglobulin-coated cells using
staphylococcus protein A-conjugated liposomes (Machy P.,
10 et al., 1988, *Proc Natl Acad Sci USA* 85:8027-8031). The
major drawback to this technology can be the expense of
mass producing ligand-conjugated liposomes.

Wu et al. report a method to target naked DNA to
specific cells. Asialoglycoprotein-DNA complexes are
15 targeted to hepatocytes expressing the asialoglycoprotein
receptor (Wu G.Y., et al., 1991, *Biotherapy* 3:87-95).
Similar to the problem encountered with immunotoxins,
however, this strategy generally limits delivery of DNA
to cells expressing receptors that are capable of DNA-
20 internalization.

Antisense DNA technology is a method for
inhibiting the expression of specific genes with
complementary DNA (Moffat, 1991, *Science* 253:510-511).
Although antisense DNA is specific in the genes that it
25 affects, it is nonspecific in the types of cells that it
gets into. This can create problems *in vivo* because it
is desirable that endogenous genes in normal cells remain
unaffected by antisense DNA (e.g., protooncogenes).
Moreover, the cost of manufacturing and administering
30 antisense DNA may be high because the phosphate moieties
of antisense DNA must be chemically modified to allow
passage through the plasma membrane, a process which
entails expensive organic chemistry. Millimolar
concentrations of antisense DNA are required to be
35 effective, posing problems of potential toxicity *in vivo*.

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Human gene therapy is therefore limited by the available technology for gene delivery. Transplantation strategies, which require surgery, limit gene therapy to an expensive service industry for a small number of
5   diseases. Targeting of genes *in situ* through local transduction is generally not precise enough. Viral vectors limit the delivery of genes *in vivo* to cells that the viruses normally infect. Liposome technologies may be infeasible because of the expense of production.
10 Simple ligand-DNA complexes will not introduce genes into cells unless the receptors, against which the ligands are directed, internalize. Accordingly, currently available gene delivery systems impose severe limitations on the spectrum of diseases that can be treated by gene therapy.
15   Summary of the Invention

The invention features a method for expressing a nucleic acid of interest in a heterologous host cell. The method involves providing a virus whose genome comprises i) the nucleic acid of interest, and ii) a
20 hybrid envelope gene. The hybrid gene encodes an envelope fragment joined to a targeting ligand, whereby the envelope fragment does not facilitate recognition or binding of its normal host cell but does facilitate efficient incorporation of the virus into a mature viral
25 particle, and whereby the targeting ligand facilitates targeting and binding of the mature viral particle to the surface of the heterologous host cell. The method also involves administering the virus so as to permit viral infection of the cell.
30   By "efficient incorporation", is meant that the hybrid envelope protein is incorporated into a mature viral particle at least 25% as frequently as the corresponding wild-type envelope protein is incorporated into a mature viral particle.

In another aspect the invention features a virus,
the genome of which encodes a hybrid envelope protein,
wherein the hybrid protein comprises an envelope fragment
joined in frame to a targeting ligand, whereby the
5   envelope fragment does not facilitate recognition or
binding of its normal host cell but which does facilitate
efficient incorporation of the hybrid envelope protein
into a mature viral particle and whereby the non-viral
protein facilitates targeting and binding of the mature
10 viral particle to the surface of a cell not normally
infected by the virus.

In a third aspect the invention features a method
for delivering a nucleic acid of interest to a
heterologous host cell. The method involves providing a
15 virus that comprises i) the nucleic acid of interest, and
ii) a hybrid envelope gene, the hybrid gene encoding an
envelope fragment joined to a targeting ligand, whereby
the envelope fragment does not facilitate recognition or
binding to its normal host cell but does facilitate
20 efficient incorporation of the virus into a mature viral
particle, and whereby the targeting ligand facilitates
targeting and binding of the mature viral particle to the
surface of the heterologous host cell. The method also
involves administering the virus so as to permit viral
25 infection of the cell.

In various preferred embodiments the virus is an
enveloped virus, preferably a Herpesviridae, a
Paramyxoviridae, or a Retroviridae, most preferably a
Moloney murine leukemia virus, or the virus may also
30 preferably be a Hepadnaviridae, a Poxviridae, or an
Iridoviridae. Similarly the virus may be a Togaviridae,
a Flaviviridae, a Coronaviridae, a Rhabodoviridae, a
Filoviridae, an Orthomyxoviridae, a Bunyaviridae, or an
Arenaviridae, or any other, yet unclassified, enveloped
35 virus.

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In other various preferred embodiments the nucleic acid of interest may include, without limitation, an antisense oncogene; a tumor suppressor gene, e.g., a gene encoding p53, or a gene encoding retinoblastoma protein
5 Rb; a toxin gene, e.g., a diphtheria toxin gene; or a gene encoding a cytokine, e.g., a tumor necrosis factor, or an interferon. The nucleic acid of interest may be either DNA or RNA, e.g., antisense DNA, or antisense RNA, or a nucleic acid encoding an antisense RNA. The nucleic
10 acid of interest may also be a gene invoking intracellular immunity, or a nucleic acid therapeutic for an inherited disease, e.g., an insulin gene, or a cystic fibrosis transmembrane regulator gene. A "gene that invokes intracellular immunity" is a gene that confers a
15 dominant negative resistant phenotype to the cell it is in, thereby protecting the cell against an invading agent.

The heterologous host cell may be a cell that has acquired mutations that result in a disease state,
20 preferably a cancer cell, e.g., a colon cancer cell. The heterologous host cell may be a cell infected with a second virus, e.g., a human immunodeficiency virus (HIV), a cell infected with an organism, or an infectious agent such as a bacterium or parasite. The infectious agent
25 may be either unicellular or multicellular. The heterologous host cell may also be a cell affected by a hereditary disease, e.g., a pancreatic beta cell, or a lung cell.

The targeting ligand, in additional various
30 preferred embodiments, may include a protein, preferably a hormone, or an immunoglobulin, more preferably an anti-tumor associated antigen-specific immunoglobulin, most preferably an anti-carcinoembryonic antigen-specific immunoglobulin, or an anti-HIVgp120 antigen-specific
35 immunoglobulin. The targeting ligand may also be a

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carbohydrate, or a lipid. The hybrid envelope fragment
may consist of a receptor binding domain, an
oligomerization domain, a transmembrane domain, a virus
budding domain, sorting signals, a signal sequence, and
5 preferably a fusion domain. In some cases the fusion
activity of the envelope fragment may be performed by a
second protein. The second protein would therefore
direct fusion of the virus with the membrane of the
targeted cell.

10   The mode of administration may include, but is not
limited to, 1) direct injection of the purified virus; or
2) implanting a container enclosing the virus into a
patient. When the virus is administered inside a
container, the virus is preferably inside a packaging
15 cell. A "packaging cell" is a cell that supplies viral
proteins necessary for production of viral vectors. By
"container" is meant a virus permeable enclosure
containing virus, or containing packaging cells with
virus therein.

20   "Normal host cell" as used herein, is a cell type
commonly infected by the naturally occurring virus. In
contrast, the term "heterologous host cell" or a
"targeted cell", as used herein, refers to a cell that is
recognized as a function of the targeting ligand portion
25 of the hybrid envelope protein, but is not recognized as
a function of the envelope portion of the hybrid envelope
protein. By "targeting ligand" is meant a molecule that
has binding affinity for a molecule on the surface of a
desired targeted cell. A "hybrid envelope protein", as
30 used herein, is a protein that includes a portion of a
viral envelope protein (or a biologically active analog
thereof) covalently linked to a targeting ligand. For
example, by a "hybrid immunoglobulin-env protein" is
meant a portion of an immunoglobulin covalently linked to
35 a portion of an envelope protein. A "hybrid envelope

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gene" is a nucleic acid that provides genetic
instructions for a hybrid envelope protein. By "hybrid
anti-carcinoembryonic antigen-specific immunoglobulin" is
meant a hybrid immunoglobulin-*env* protein that

5 specifically binds to a carcinoembryonic antigen.

The term "fragment", as applied to an envelope
protein fragment, includes some but not all of the
envelope protein. A fragment will ordinarily be at least
about about 20 amino acids, typically at least about 30

10 amino acids, usually at least about 40 contiguous amino
acids, preferably at least about 50 amino acids, and most
preferably at least about 60 to 80 or more contiguous
amino acids in length. Fragments of an envelope protein
can be generated by methods known to those skilled in the

15 art (e.g., those described herein).

A biologically active fragment of a viral envelope
protein is one that possesses at least one of the
following activities: a) it can bind to a cell membrane
if given the appropriate targeting ligand; b) it can

20 enable fusion with a cell membrane; or c) it can enable
incorporation of proteins into a mature viral particle.
These three biological activities can be performed by the
same envelope protein fragment, or by two separate
envelope protein fragments. As stated above, the

25 envelope fragments of this invention do not facilitate
recognition or binding of the virus' normal host cell.
This is accomplished by either destroying the activity of
the normal receptor binding region by mutation, or by
physically deleting it. A new recombinant receptor-

30 binding region is added in its place. The ability of a
candidate fragment to exhibit a biological activity of a
viral envelope protein can be assessed by methods known
to those skilled in the art.

The envelope fragment may include the amino acid

35 sequence of a naturally-ocurring viral envelope or may be

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a biologically-active analog thereof. The biological
activity of an envelope analog is assessed using the
methods described herein for testing envelope fragments
for activity.

5   Applicants have provided an efficient and reliable
means for specifically delivering therapeutic genes or
antisense nucleic acids to particular animal, plant or
human cell types, or to cells of infectious agents.
Their method facilitates treatments for mutagenically
10 acquired, infectious, or inherited diseases, e.g., by
either 1) antagonizing the effect of an existing cellular
gene; 2) complementing the defect of an existing cellular
gene; 3) destroying the target cells through the
introduction of new genetic material; or 4) changing the
15 phenotype of the target cells through the introduction of
new genetic material. To specifically target cells for
delivery, a hybrid envelope protein (e.g., an envelope-
antibody or envelope-ligand hybrid) is utilized which
directs specific interaction with a particular target
20 host cell. The viruses itself, through its efficient
internalization mechanisms, facilitates efficient uptake
of the therapeutic gene. Such viral vectors are uniquely
adapted to deliver genes, RNA, or drugs to cell surface
proteins that do not normally internalize.

25   Another advantage of this invention is that it
overcomes the problem of gene regulation encountered with
other methods of gene therapy. Genetically-altered cells
must not only synthesize the gene products at the right
location, at the right time, and in the right amounts,
30 but must also be regulated in the same manner as the
indigenous tissue. That is, the transduced cells must
also have all the proper signal transduction mechanisms
to respond to extracellular signals. This may be a
problem in gene therapy for diabetes, for example, where
35 transplanting fibroblasts with insulin genes can be

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ineffective or even harmful. As fibroblasts do not
contain the same receptors and signal transduction
machinery as pancreatic beta cells, the insulin genes may
be expressed differently. Targeting genes to the right
5 cells insures that the genes will be properly regulated.

        In an additional aspect of the invention, a
selection scheme is devised for creation of hybrid
envelope protein-containing viruses. This strategy will
be feasible for env proteins fused with immunoglobulins
10 or with any ligand that recognizes specific receptors on
cells.

        Other features and advantages of the invention
will be apparent from the following description of the
preferred embodiments thereof, and from the claims.

15   Detailed Description

        The drawings will first briefly be described.

Drawings

        FIG. 1 is a representation of a scheme for
constructing retroviral vector pLNCX*.

20   FIG. 2 is a representation of a scheme for
constructing plasmid LNCenvpA.

        FIG. 3 is a representation of a scheme for
constructing plasmid LNCenv.

        FIG. 4 is a representation of a scheme for
25 constructing plasmid pUC Star-Sig.

        FIG. 5 is a representation of a scheme for
constructing plasmid LNC-Sig.

        FIG. 6 is a representation of a scheme for
constructing plasmid LNC-antiCEA.

30   FIG. 7 is a representation of plasmids used in the
construction of targeted viruses.

        FIG. 8 is a representation of a strategy for
generating targeted retroviruses involving construction
of hybrid immunoglobulin-env genes in vitro.

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FIG. 9 is a representation of a strategy for generating targeted retroviruses involving generation of pooled virus constructions.

FIG. 10 is a representation of a strategy for
5 generating targeted retroviruses involving selection and characterization of targeted virus.

FIG. 11 is a representation of a plasmid containing a targeted retroviral vector.

FIG. 12 is a representation of a scheme for
10 constructing plasmid pUC Star-antiCEA.

FIG. 13 is a representation of an alternative scheme for constructing plasmid LNC-*anti*CEA.

What follows is a procedure for the delivery of genes to target cells using targeted viral vectors. To
15 create and target a virus, the receptor recognition domain of the viral envelope protein is replaced with a ligand directed against a specific cell surface receptor. The hybrid envelope protein is incorporated into the viral envelope during the budding process, producing a
20 hybrid virus *in vivo*. Upon infection of a host, the hybrid virus specifically recognizes its target cell and resultant fusion with that cell facilitates internalization (into the target cell) of viral genes, including the therapeutic gene(s) which are engineered
25 into the viral genome. Such internalization can be extremely important; for example, immunotoxins, although efficient at delivering toxin molecules to target cells, are often clinically ineffective since the cell surface molecules to which they are targeted do not internalize,
30 and internalization is required for entry of the toxin molecules into the cells (Waldmann, T.A., 1991, *Science* 252: 1657-1662). Targeted viruses circumvent this requirement for receptor internalization since the virus itself contains the necessary cell fusion machinery
35 (Gilbert, J.M., et al., 1990, *J Virol* 64: 5106-5113;

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Roizman, B. et al., 1990, in BN Fields, et al., eds.
Virology, Raven Press, Ltd. New York).

General Requirements for Targeted Viruses

In general targeted viruses are constructed by
5 replacing the receptor recognition domain of the viral
envelope protein with a ligand directed against a
specific cell surface receptor. The ligand can be,
without limitation, an immunoglobulin (e.g., FAb, dAb,
Fd, or Fc), a hormone, or any other synthetic or natural
10 protein that can direct the binding of the targeted
viruses to a cell surface molecule. The ligand is
biologically incorporated into the viral envelope by
genetic fusion with that portion of the normal viral
envelope protein involved in viral assembly and budding.
15 The envelope portion of the hybrid protein consists of an
envelope fragment (or analog thereof) that is sufficient
to direct efficient incorporation of the envelope hybrid
protein into the viral envelope. Preferably, the
envelope hybrid protein no longer directs an interaction
20 between the virus and its normal host cell.

It has been demonstrated that changing the
receptor specificity of the envelope protein of a virus
changes the virus's tropism. For example, vesicular
stomatitis virus (VSV) pseudotypes that have their virus
25 envelope replaced with that of a retrovirus acquire the
ability to infect retrovirus infectable cells (Schnitzer,
T.J., et al., 1977, *J Gen Virol* 23:449-454; Zavada. J.,
et al., 1972, *J Gen Virol* 15:183-191), indicating that
species specific protein-protein interactions between a
30 virus core protein and an envelope protein are not
critical for virus fusion and penetration at least in
these cases. Moreover, previous experiments have also
indicated that virus envelopes can tolerate changes in
length or conformation introduced into the envelope
35 protein, e.g., by a conjugated ligand. For example,

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Gitman *et al.* have shown that Sendai virus envelopes
reconstituted with viral envelope glycoproteins,
chemically cross-linked to anti-erythrocyte antibodies
acquire the ability to bind to erythrocytes that had been
5   stripped of the normal virus receptor. Similarly, Sendai
virus envelopes reconstituted with envelope proteins,
chemically cross-linked to insulin molecules, were able
to bind to receptor-stripped erythrocytes expressing the
insulin receptor. In both cases, envelope binding but
10 not fusion occurred with the receptor-stripped
erythrocytes. Fusion between the conjugated envelopes
and erythrocytes occurred, however, when the conjugated-
envelopes were coreconstituted with the normal viral
hemagglutinin/neuraminidase and fusion proteins (Gitman,
15 A.G., et al., 1985, *Biochem* 24:2762-2768).
        Preferably, the targeted virus contains 1) a viral
envelope derived from a host cellular membrane; 2) a
transmembrane hybrid envelope protein that directs the
binding and penetration of the virus to specific target
20 cells; 3) a transmembrane envelope protein that directs
the fusion of the targeted virus with the cellular
membrane of the targeted cell for viral penetration
(e.g., the targeting protein itself or another envelope
protein); 4) viral core proteins; 5) a foreign gene(s) of
25 interest; and 6) all necessary viral and genetic
components for penetration and expression of genes
contained in the viral genome. The transmembrane hybrid·
envelope protein consists of 1) determinants that enable
the hybrid protein to become processed and incorporated
30 into viral envelopes; 2) determinants that enable fusion
of the viral envelope with the targeted cellular
membrane; these are essential for penetration of the
targeted virus; 3) a ligand determinant that enables the
targeted virus to recognize and bind to specific
35 receptors on target cells. The viral genome may also

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include bacterial selectable markers (e.g., ampicillin
resistance) and/or a mammalian cell selectable marker
(e.g., neomycin resistance).

The transmembrane hybrid protein is constructed
5 genetically by splicing the cell surface receptor binding
domain of a ligand gene to a portion of the viral
envelope protein gene. The transmembrane hybrid protein
must retain those portions of the envelope protein that
direct the efficient post-translational processing,
10 sorting and incorporation of the protein into the viral
envelope.

The following domains must be considered in
constructing the hybrid ligand-envelope protein:
1. The receptor binding domain

15   The receptor binding domain is that portion of the
envelope protein that recognizes and binds to cell
surface receptors. In hybrid envelope proteins, this
portion of the envelope protein is replaced with ligand
sequences. The receptor binding domain of retrovirus
20 envelope proteins has been localized to the SU subunit
(Coffin, J.M., 1990, in BN Fields, et al., eds. Virology,
Raven Press, Ltd., New York). Since the SU protein of
retroviruses is coded for 5' to the transmembrane
protein, replacement of the amino-terminal sequences of
25 the envelope protein with ligand sequences poses no
problem for the creation of a functional hybrid ligand-
envelope protein.
2. Proteolytic cleavage site

The envelope protein of retroviruses is
30 synthesized as a polyprotein which is later
proteolytically cleaved to form SU and TM heterodimers.
In construction of the hybrid ligand-envelope protein,
the proteolytic cleavage site should be eliminated. The
proteolytic cleavage site should be eliminated either by
35 deletion or by site directed mutagenesis. Perez and

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Hunter have demonstrated that elimination of the
proteolytic cleavage site does not block transport or
surface expression of Rous sarcoma virus envelope
proteins (Perez, L.G., et al., 1987, *J Virol* <u>61</u>:1609-
5 1614).

   3. Oligomerization domain

         The envelope proteins of many animal viruses
associate to form trimers (Fields, B.N., et al., 1991,
<u>Virology</u>, 2nd ed., Raven Press, Ltd., New York).
10 Trimerization of the envelope protein is thought to be
essential for the proper transport and insertion of
envelope proteins into the viral envelope (Singh, J. et
al., 1990, *Embo J* <u>9</u>:631-639; Kreis, T.E., et al., 1986,
Cell <u>46</u>:929-937). Therefore it is important that this
15 domain be retained in the hybrid ligand-envelope protein.
The trimerization domain likely resides in the
transmembrane TM protein of retroviruses (Einfeld, D., et
al., 1988, *Proc Natl Acad Sci USA* <u>85</u>:8688-8692); hence,
creation of a functional hybrid ligand-envelope
20 retroviral protein lacking the SU subunit is possible.
Some viral envelope proteins may oligomerize to form
stoichiometric combinations other than trimers.

   4. Fusion domain

         The fusion domain is a hydrophobic stretch of
25 amino acids that is involved in fusion of the virus
envelope with the cell membrane (Wiley, D.C., et al.,
1990, in Fields, B.N. et al., eds. <u>Virology</u>, 2nd ed.,
Raven Press, Ltd., New York). Viral fusion allows entry
of the viral core proteins and genome into the cell. In
30 influenza virus, the fusion domain, located in the amino
terminus of the envelope HA2 protein, is sequestered in
the hemagglutinin trimer until a low pH-induced
conformational change allows presentation of the fusion
domain to the cell membrane. Trimerization of the
35 envelope proteins can prevent constitutive expression of

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fusion activity by sequestering it within an internal
hydrophobic pocket. A potential fusion domain has been
located within the extracellular portion of the gp37 TM
protein of Rous sarcoma virus (Hunter E. et al., 1983, *J*
5 *Virol* 46:920-936). Similar hydrophobic fusion sequences
have been noted in the p15E protein of Moloney murine
leukemia virus (Mo-MuLV) (Chambers, P., et al.,1990, *J*
Gen Virol 71:3075-3080).

        In constructing a hybrid ligand-envelope protein,
10 it may be necessary to eliminate the fusion domain to
prevent the possibility of constitutive fusion activity,
a state that may impair the infectivity of targeted
viruses. Therefore two proteins may be incorporated into
the viral envelope of targeting viruses. The first
15 protein is the hybrid ligand-envelope protein which
directs targeting of the virus but lacks fusion activity.
The second protein is an envelope protein possessing
fusion activity but lacking a receptor binding domain.
This type of situation is observed for paramyxoviruses
20 where one envelope protein is dedicated to targeting
while another carries out fusion (Kingsbury, D.W., 1990,
B.N. Fields, et al., eds. Virology, 2nd ed., Raven
Press, Ltd., New York.). Where it is not necessary to
prevent constitutive fusion activity, both activities may
25 be included in one protein.
5. Transmembrane domain
        The transmembrane domain is a stretch of
approximately twenty or more amino acids that anchor the
envelope protein to the viral envelope. It is located
30 within the p15E protein of Moloney murine leukemia virus
(Chambers, et al., supra). Retention of the
transmembrane domain is thought to be essential since
deletion of the transmembrane domain results in secretion
of the synthesized envelope protein (Perez, L.G., et al.,
35 1987, *J Virol* 61:2981-2988).

6. Virus budding domain

       Amino acid sequences within the envelope protein
may be involved with the exclusive incorporation of viral
envelope proteins into viral envelopes and with virus
5 budding. The virus budding domain directs the hybrid
ligand-envelope protein into the viral envelope. These
sequences are thought to reside within the portion of the
envelope protein facing the inside of the virus and may
involve specific protein-protein interactions between
10 envelope proteins and viral core or matrix proteins.
Although Perez et al. demonstrated that deletion of the
carboxy-terminal sequences of the Rous sarcoma virus env
protein resulted in normal budding of the mutant virus
(Perez, L.G., et al., 1987, J Virol 61:2981-2988),
15 evidence exists that, for other viruses, interactions
between envelope proteins and viral core proteins may
direct virus assembly and envelopment (BN Fields, et al.,
eds., 1990, Virology, Raven Press, Ltd., New York).

7. Sorting signals and other signals

20   Sorting signals are determinants that direct the
envelope protein to the correct intracellular location
during post-translational processing. These sequences
insure that the envelope protein passes through the
endoplasmic reticulum, Golgi apparatus, and other
25 organelles until it eventually reaches the viral
envelope. Other signals that may have to be retained in
the hybrid ligand-envelope protein are glycosylation
sequences and sequences involved in effective
conformation of the envelope protein (e.g., disulfide
30 bonds).

8. Signal sequence

       The signal sequence is an amino-terminal
hydrophobic stretch of amino acids that directs the
envelope protein into the endoplasmic reticulum. The
35 signal sequence, which is later proteolytically cleaved,

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is essential for the hybrid ligand-envelope protein to become located in a membrane.

The diversity of signals and domains that must be considered in constructing targeted viruses requires that
5 precise and correct splicing of ligand and envelope genes occur. The present invention describes a selection scheme for constructing targeted viruses whereby the ligand gene is spliced to an envelope gene fragment; this hybrid gene codes for those portions of the envelope
10 protein which are required to direct efficient incorporation of the resultant hybrid envelope-ligand protein into the mature viral particle. According to the selection scheme, cell surface receptor binding domains of ligand genes are randomly ligated to progressive
15 deletions of viral envelope genes. The correct combination of ligand and envelope sequences is determined by a selection scheme for the production of biologically active targeted virus. The selection scheme not only produces targeted virus but simplifies the
20 construction of future targeted viruses.

A Specific Example of a Targeted Retrovirus

There now follows an example of a recombinant retrovirus which targets and infects particular host cells for the purpose of delivering to those cells a
25 desired therapeutic gene. This example is provided for the purpose of illustrating, not limiting, the invention.

Moloney murine leukemia virus (Mo-MuLV) is a mouse ecotropic retrovirus. A recombinant Mo-MuLV based retroviral vector that is targeted to colon cancer cells
30 is constructed. The targeted retroviral vector delivers the neomycin resistance gene to colon cancer cells. Targeting to human colon cancer cells is accomplished by incorporating into the viral envelope hybrid immunoglobulin-*env* proteins directed against
35 carcinoembryonic antigen. Carcinoembryonic antigen (CEA)

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is a tumor associated antigen expressed on the surface of
human colon cancer cells but not on the surface of normal
adult cells. The CEA glycoprotein, possessing multiple
membrane spanning alpha helices, does not internalize in
5 response to ligand (Benchimol, S. et al., 1989, *Cell*
57:327-334). A protein that is homologous to
carcinoembryonic antigen has recently been shown to be
the receptor for mouse hepatitis virus (Dveksler, G.S.,
et al, 1991, *J Virol* 65:6881-6891).
10   For the purpose of this illustration, a single
variable region of the heavy chain of anti-CEA is fused
to a portion of the *env* gene. Single variable heavy
chain fragments (dAb) have been shown to be as effective
in antigen binding as fragmented antibodies (FAb),
15 containing both heavy and light chain fragments, and
intact monoclonal antibodies (Ward, E.S., et al., *Nature*
341:544-546). The function of immunoglobulin-*env*
proteins is not limited, however, to the use of dAb's and
can be applied with FAb's, Fv's and mAb's.
20 <u>Modification of the retroviral vector LNCX</u>
        LNCX is a Moloney murine leukemia virus based
retroviral vector contained in the plasmid pLNCX (Miller,
A.D., et al., 1989, *Biotechniques* 7:980-990). pLNCX
contains a unique HindIII and ClaI cloning site for
25 expression of inserted genes, a cytomegalovirus (CMV)
promoter, a polyadenylation site (pA), retroviral long
terminal repeats (LTR) for retroviral RNA transcription
and reverse transcription, a bacterial neomycin
resistance gene (Neo) which conveys resistance to both
30 neomycin and G418, a bacterial origin of replication
(Or), a bacterial ampicillin resistance gene (Amp), and a
retroviral RNA packaging sequence (ψ+). LNCX is modified
to contain a unique SalI site as shown in Figure 1.
pLNCX is linearized with XbaI and subcloned into the XbaI
35 site of the phagemid BluescriptII SK+ (Stratagene, La

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Jolla, CA). Single stranded DNA is purified and the
unique BstEII site of LNCX is converted into a SalI site
by site directed mutagenesis with the oligonucleotide
5'-GCAGAAGGTCGACCCAACG-3' (SEQ ID NO: 1). The BstEII
5   site is located within the extended packaging signal (Ψ+)
of Mo-MuLV RNA (Bender, M.A., et al., 1987, *J Virol*
<u>61</u>:1639-1646; Adam, M.A., et al., 1988, *J Virol* <u>62</u>:3802-
3806; Armentano, D. et al., 1987, *J Virol* <u>61</u>:1647-1650).
Conversion of the BstEII site to SalI does not affect
10   packaging since this region has been determined to be
dispensable for efficient packaging (Schwartzberg, P., et
al., 1983, *J Virol* <u>46</u>:538-546; Mann R. et al., 1985, *J
Virol* <u>54</u>:401-407; and Mann, R., et al., 1983, *Cell*
<u>33</u>:153-159). The BstEII site is converted into a SalI
15   site because BstEII sites, but not SalI sites, frequently
occur in heavy chain genes (Chaudhary, V.K., et al, 1990,
Proc Natl Acad Sci USA <u>87</u>:1066-1070). The SalI
containing plasmid is recircularized with XbaI and DNA
ligase to form the plasmid pLNCX*.
20   <u>Cloning of the Mo-MuLV env protein in pLNCX*</u>
          The Mo-MuLV *env* gene is cloned into pLNCX* as
shown in Figure 2. The Mo-MuLV *env* gene is excised from
plasmid p8.2 (Shoemaker, C., et al., 1980, *Proc Natl Acad
Sci USA* <u>77</u>:3932-3936) as a 1.9kb ScaI-NheI fragment. The
25   1.9kb ScaI-NheI fragment contains the entire coding
region for the p15E transmembrane protein and the
majority of the coding region for the gp70 SU protein.
The 5'-protruding ends are digested with S1-nuclease, and
HindIII linkers (5-CCAAGCTTGG-3'; SEQ ID NO: 2) are
30   added. The *env* gene is cloned as a HindIII fragment in
the HindIII site of pLNCX* to form plasmid LNCenvpA. The
orientation of the HindIII *env* fragment is such that it
can be transcribed and expressed from the cytomegalovirus
(CMV) promoter.

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Modification of LNCenvpA to LNCenv

LNCenvpA is cloned as an XbaI fragment in phagemid pBluescript II SK+ for additional site directed mutagenesis (Figure 3). The env encoding HindIII
5 fragment contains a polyadenylation signal that may interfere with the polyadenylation signal provided by the viral vector. The AAUAAA polyadenylation signal is therefore changed to AAGAAA by site directed mutagenesis with the oligonucleotide 5'-GTTTTCTTTTATC-3' (SEQ ID NO:
10 3). The HindIII site located at the 3' end of the env gene is eliminated by site directed mutagenesis with the oligonucleotide 5-CAAGCATGGCTTGCC-3' (SEQ ID NO: 4). The env containing retroviral vector is recircularized by XbaI restriction and ligation to form plasmid LNCenv.
15 Molecular cloning of anti-CEA immunoglobulin genes

cDNA encoding the mature variable region domain of anti-CEA heavy chain genes is cloned as an XhoI-SpeI fragment using the polymerase chain reaction (PCR) and RNA template. RNA is derived from the spleen of mice
20 immunized against purified carcinoembryonic antigen. Alternatively, RNA can be derived from hybridoma cell lines that secrete monoclonal antibodies against CEA, e.g., 1116NS-3d (American Type Culture Collection CRL8019) or CEA 66-E3 (Wagener, C., et al., 1983, J
25 Immunol 130:2308-2315).

The following PCR primers hybridize to cDNA encoding the aminoterminal end of mature heavy chain genes (Stratacyte, Inc.). The degenerate primers introduce an XhoI site which is underlined.

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```
5' AGGTGCAGCTGCTCGAGTCGGG 3'  (SEQ ID NO: 5)
5' AGGTGCAACTGCTCGAGTCGGG 3'  (SEQ ID NO: 6)
5' AGGTGCAGCTGCTCGAGTCTGG 3'  (SEQ ID NO: 7)
5' AGGTGCAACTGCTCGAGTCTGG 3'  (SEQ ID NO: 8)
5' AGGTCCAGCTGCTCGAGTCTGG 3'  (SEQ ID NO: 9)
                XhoI
```

The following PCR primer hybridizes to
immunoglobulin heavy chain mRNA within the region coding
for the J-region and introduces SpeI and BstEII sites.

```
      5' CTATTAACTAGTGACGGTTACCGTGGTCCCTTGGCCCCA 3' (SEQ
ID NO: 10)
           SpeI     BstEII
```

The amplified anti-CEA variable heavy chain DNA is
cloned as an XhoI-SpeI fragment in an ImmunoZAP H vector
(Stratacyte, Inc.) (Mullinax, R.I. et al., 1990, *Proc
Natl Acad Sci USA* 87:8095-8099). ImmunoZAP H is a
modified lambdaZAP vector that has been modified to
express in E.coli immunoglobulin variable heavy chain
fragments behind a pelB signal sequence. The procedure
could similarly be performed by expressing immunoglobulin
variable light chain fragments in a packaging cell line.
Identification of high affinity anti-CEA clones

Clones expressing high affinity anti-CEA
antibodies are identified by a filter binding assay. The
anti-CEA phage library is screened by nitrocellulose
plaque lifts with [^{125}I]bovine serum albumin conjugated to
CEA, as previously described (Huse, W.D., et al., 1989,
Science 246:1275-1281). High and intermediate affinity
anti-CEA clones are chosen for further manipulation.
Construction of plasmid LNC-*immunoglobulin*

Two strategies are presented for creating plasmid
LNC-*immunoglobulin* (in this example, LNC-*antiCEA*, which
codes for an anti-CEA immunoglobulin gene). LNC-
immunoglobulin vectors encode an immunoglobulin peptide

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fused to an amino-terminal signal sequence. Some amino acids at the amino-terminal end of the mature immunoglobulin peptide have been modified by the PCR primers used to generate the immunoZAP library.

5   Moreover, the design of the LNC-antiCEA plasmid results in the insertion of an extra amino acid at the amino terminal end. These amino acid changes do not affect antigen binding because 1) the amino acid changes are conservative; 2) the affected amino acids are normally

10   variable at those sites; and 3) the affected amino acids occur within the framework region of immunoglobulins which has been shown not to participate in antigen binding or conformation of the antibody (Relchman et al. 1988 *Nature* 332:323-327). It is for these same reasons

15   that cleavage of the signal sequence from mature peptide will not be affected.

Both strategies for creating LNC-*immunoglobulin* rely on the use of plasmid pUC Star-Sig, the construction of which is presented below.

20   An immunoglobulin signal sequence is cloned into a modified pUC119 vector to create pUC Star-*Sig* as follows (Figure 4). pUC119 is a phagemid containing a polylinker cloning site. The multiple cloning sites of pUC119 are replaced with new restriction sites by insertion of the

25   following polylinker into the HindIII and XbaI sites of pUC119.

```
HindIII   PstI   XhoI   BclI   SpeI   NotI      ClaI   XbaI
5'-AGCTTCTGCAGGCTCGAGTGATCAACTAGTGCGGCCGCATCGATT-3'  (SEQ
ID NO: 11)
```

30   ```
3'-AGACGTCCGAGCTCACTAGTTGATCACGCCGGCGTAGCTAAGATC-5' (SEQ
ID NO:12)
```

The modified pUC119 is called pUC Star-1 (Figure 4). The restriction sites may be further separated by small

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linkers, if adjacent restriction sites interfere with one
another during digestion.

  The signal sequence from an anti-NP immunoglobulin
heavy chain gene is isolated from plasmid pcDFL.1 (Ucker,
5 D.S., et al., 1985, *J Immnol* <u>135</u>:4204-4214) as a ~330bp
PstI fragment. The 330bp PstI fragment is subcloned into
pUC Star-1 to yield plasmid pUC Star-*Sig* (Figure 4). The
PstI fragment is oriented so that the signal sequence can
be expressed.

10   <u>A. Construction of LNC-*immunoglobulin* through</u>
<u>plasmid LNC-*Sig*, an immunoglobulin expression vector.</u>

  LNCX* is converted into a eukaryotic
immunoglobulin expression vector (Figures 4 and 5). An
immunoglobulin heavy chain signal sequence and XhoI-SpeI
15 cloning sites are inserted behind the CMV promoter of
plasmid LNCX* to allow expression of the PCR amplified
immunoglobulin genes. Conversion of LNCX* is as follows.

  The immunoglobulin heavy chain signal sequence is
recovered from pUC Star-*Sig* as a HindIII-ClaI restriction
20 fragment and cloned into the HindIII-ClaI sites of LNCX*.
The resulting plasmid, LNC-*Sig* contains a retroviral
vector with the immunoglobulin heavy chain signal
sequence under control of the CMV promoter (Figure 5).

  An anti-CEA gene from the immunoZAP library is
25 then subcloned into LNC-*Sig* to form plasmid LNC*anti*-CEA.
This generates an anti-CEA variable heavy chain gene
containing a signal sequence (Figure 6). The anti-CEA
gene is first excised from immunoZAP phage DNA as a
Bluescript SK- phagemid (see lambdaZAP protocols,
30 Stratagene, Inc. La Jolla, CA). The anti-CEA gene is
purified as an XhoI-SpeI fragment and ligated to XhoI-
SpeI restricted LNC-*Sig*. LNC-*Sig* contains three SpeI
sites. Therefore, to generate plasmid LNCanti-CEA, the
ligation mix is transformed into neomycin-sensitive,
35 ampicillin-sensitive <u>E. coli</u> and neomycin-resistant,

ampicillin-resistant transformants are selected for.
Plasmid LNC*anti*-CEA is screened from neomycin-resistant,
ampicillin-resistant transformants by using the SpeI-XhoI
anti-CEA restriction fragment from Bluescript SK-anti-CEA
5    as a probe.   The SpeI site is used because of dependence
upon the available sites in the ImmunoZap expression
vector.   To simplify construction of LNC*anti*-CEA, a
unique NotI site can be introduced into the ImmunoZap H
expression vector so that NotI sites can be used instead
10   of SpeI sites.

B.  Construction of LNC-*immunoglobulin* through
plasmid pUC Star-*Sig*

An anti-CEA gene from the immunoZAP library is
subcloned into plasmid pUC Star-*Sig* to form plasmid pUC
15   Star-anti-CEA.   This generates an anti-CEA variable heavy
chain gene containing a signal sequence (Figure 12).   The
anti-CEA gene is excised from immunoZAP phage DNA as a
Bluescript SK-phagemid (see lambdaZAP protocols,
Stratagene, Inc., La Jolla, CA).   Bluescript SK-anti-CEA
20   double stranded DNA is prepared and restricted with XhoI
and SpeI.   The anti-CEA containing XhoI-SpeI fragment is
purified by electroelution and ligated to Xho-SpeI
restricted pUC Star-Sig to create plasmid pUC Star-
antiCEA (Figure 12).

25        The antiCEA gene is transferred from pUC Star-anti
CEA to LNCX* as a HindIII-ClaI fragment to create plasmid
LNC-antiCEA (figure 13).   The antiCEA-containing HindIII-
ClaI fragment is purified from pUC Star-antiCEA by
electroelution.   Phosphatase treated, HindIII-ClaI
30   restricted LNCX* is ligated with the purified HindIII-
ClaI antiCEA fragment to generate LNC-antiCEA (figure
13).

Strategy for generating targeted viruses

The starting materials for generation of targeted
35   viruses are the LNC*env* and LNC-immunoglobulin (in this

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example, LNC-*anti*CEA) plasmids shown in Figure 7. ·
Figures 8-11 diagram the general principle for the
primary generation of targeted viruses.  Hybrid
immunoglobulin-*env* proteins are generated that target
5   viruses to cells expressing carcinoembryonic antigen.
Since the location of important determinants for envelope
protein sorting (S), trimerization (T), and fusion (F) is
not known with certainty, the immunoglobulin gene is
ligated to progressive deletions of the *env* gene and
10  functional immunoglobulin-*env* hybrids are selected for.
Useful Envelope Fragments or Analogs

        The envelope portion of the fusion protein may
consist of any portion of the envelope protein (or any
analog thereof) which is sufficient to direct efficient
15  incorporation of the envelope fusion protein into the
viral coat (upon budding of the recombinant virus from a
producer cell line).  Such fragments or analogs may be
determined using the following general selection scheme
which generally involves ligation of cell surface
20  receptor binding domains of ligand genes to progessive
deletions of viral envelope genes.  The correct
combination of ligand and envelope sequences is
determined by a selection scheme for the production of
biologically active targeted virus.  The selection scheme
25  not only produces targeted virus but simplifies the
construction of future targeted viruses.
Construction of hybrid immunoglobulin-*env* genes *in vitro*

        Plasmid LNC*env* contains the coding region for the
Mo-MuLV *env* polyprotein (Figure 8).  LNC*env*  is first
30  linearized by HindIII restriction.  A range of deletions
extending into the *env* gene is created by collecting
aliquots of Exonuclease III treated DNA over time and
removing 5'-processive ends with S1-nuclease (Guo, I.H.,
et al., 1983, *Methods Enzymol* 100:60; and Sambrook, J.,
35  et al., 1989, Molecular Cloning, Cold Spring Harbor

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Laboratory Press, Cold Spring Harbor). NotI linkers (5'
AGCGGCCGCT 3' SEQ ID NO: 13) are ligated onto the blunt
end termini and restricted with NotI. This results in a
NotI restriction overhang at the 5'-border of every
5   deletion within the *env* gene. The NotI overhangs at the
other end of the molecules are removed by SalI
restriction of the reaction mixture. The reaction
mixture is then treated with phosphatase to prevent
circularization of the reaction products.
10        The reaction mixture is ligated to a SalI-NotI
restriction fragment from LNC-*anti*CEA that contains the
anti-CEA variable heavy chain gene. This creates a pool
of functional retroviral vectors encoding an anti-CEA
peptide fused to a series of *env* deletions.
15  <u>Generation of pooled virus constructions</u>
          The total reaction mixture from above is
transformed into ampicillin-sensitive <u>E. coli</u> and
ampicillin resistance is selected for (Figure 9).
Recombinants containing functional retroviral vectors are
20  selected for since only they contain the ampicillin
resistance gene. Plasmid DNA is prepared from
transformants grown in liquid culture to create a pool of
retroviral vectors containing different immunoglobulin-
*env* fusion genes.
25        The DNA is transfected into the crip2 retroviral
packaging cell line (Danos, O., et al., 1988, *Proc Natl
Acad Sci USA* <u>85</u>: 6460-6464). Alternatively, DNA is
transfected into a packaging cell line that does not
encode wild-type env protein. The transfected packaging
30  cell line synthesizes each of the different hybrid
immunoglobulin-*env* proteins as well as the wild type *env*
protein (encoded by an *env* gene contained in the cell
line). The transfected packaging cell line secretes a
pool of enveloped retroviruses containing the different
35  retroviral genomes encoding hybrid immunoglobulin-*env*

genes.  If the hybrid immunoglobulin-*env* protein retained
all of the necessary determinants for efficient
incorporation into viral envelopes then the hybrid-*env*
protein can be incorporated into viral envelopes.  Wild
5  type *env* proteins encoded for by the packaging cell line
are also incorporated into the viral envelopes.  This
creates a virus containing both wild type and hybrid *env*
proteins in the viral envelope.  This system therefore
selects for immunoglobulin-*env* hybrids that can
10  incorporate their gene products into the viral envelope.
Virus pools are harvested from media filtered at
0.45μ to remove contaminating G418-resistant packaging
cells.

Selection and characterization of targeted virus

15           G418-sensitive target cells are exposed to virus
pools by standard procedures, and G418-resistant cells
are selected for.  The target cells can be any non-mouse
cell line (uninfectable by wild type Mo-MuLV) that
expresses carcinoembryonic antigen.  Examples include
20  ATCC COLO 205, a human cell line isolated from the
ascites of a patient with carcinoma of the colon
(A.T.C.C.#CCL 222); LR-73 CEA, a chinese hamster ovary
cell line transfected with a mouse carcinoembryonic
antigen gene (Benchimol, S. et al., *supra*); and HCT48, a
25  human colon adenocarcinoma cell line (Shi, Z.R., et al.,
1883, *Cancer Res* 43:4045-4049).

G418-resistant cells can only have arisen from
transduction of the neomycin resistance gene by targeted
virus.  This system therefore selects for recombinant
30  viruses that have hybrid immunoglobulin-*env* proteins that
have retained all the necessary determinants for viral
targeting and fusion.

Rescue of integrated immunoglobulin-*env* gene

Infection by targeted virus results in integration
35  of the hybrid envelope gene that created the targeting

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protein.  The integrated hybrid immunoglobulin-*env*·gene
is rescued from the host DNA by polymerase chain reaction
(PCR) with the following primers:

PCR 5' Rescue primer:
5  5'-CCAGCCTCCGCGGCCCCAAGCTTCTGCA-3' (SEQ ID NO: 14)
                        HindIII
PCR 3' Rescue primer:
5'-GGTTCTCTAGAAACTGCTGAGGGC-3' (SEQ ID NO: 15)
          XbaI


10        PCR amplification with these primers generates the
immnoglobulin-*env* gene bordered by HindIII and XbaI
sites.  The amplified DNA is restricted with HindIII and
XbaI to create sticky ends and the DNA is ligated into
HindIII-XbaI cut LNCX*.  When transfected into crip2
15  packaging cells, this generates a retroviral vector
targeted to cells expressing cell surface
carcinoembryonic antigen (e.g., colon cancer cells).
        The retroviral vector produced in the above
selection scheme is targeted to both CEA-expressing human
20  cells (directed by the hybrid envelope protein) and
normal mouse cells (directed by the wild type envelope
protein) when produced in crip2 packaging cells.  To
create viruses that infect target cells only, the
retroviral vector will first be tested to determine if
25  incorporation of the hybrid envelope protein alone is
sufficient to direct virus fusion.  This is accomplished
by transfecting DNA into a modified packaging cell line
that does not encode wild type *env*.
        If fusion functions are found to have been
30  supplied from the wild type envelope protein, targeted
viruses will be created as follows.  A packaging cell
line will be created that encodes an *env* gene containing
mutations in the receptor binding domain.  When

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transfected with the targeted viral vector DNA, targeted
viruses expressing both hybrid ligand-*env* proteins and
*env* proteins with mutated binding sites will be produced.
The viruses will exclusively infect target cells.

5  The targeted viral vector is a universal vector

        The viral vector that is constructed by the above
procedure is a universal targeted vector (Figure 11).
Targeting to other cells is accomplished by replacing the
XhoI-SpeI anti-CEA fragment with any XhoI-SpeI fragment

10  encoding an in-frame immunoglobulin or ligand directed
against specific cell surface proteins.  For example, an
XhoI-SpeI immunoglobulin-containing fragment from an
immunoZAP library can be fused in frame behind a signal
sequence and subcloned into LNCX* through the pUC Star-

15  Sig plasmid, as outlined above.  Substituting a SalI-NotI
fragment from another LNC-immunoglobulin plasmid into the
universal vector would create another targeted virus
vector.

Other Viral Vectors

20        Any enveloped virus may be used as a vector for
the targeted delivery of a therapeutic gene.  Particular
examples include both DNA and RNA viruses, such as
Herpesviridae, e.g., herpes simplex type 1 or 2,
Paramyxoviridae, Retroviridae, Hepadnaviridae,

25  Poxviridae, Iridoviridae, Togaviridae, Flaviviridae,
Coronaviridae, Rhabodoviridae, Filoviridae,
Orthomyxoviridae, Bunyaviridae, or Arenaviridae, or any
other, yet unclassified, enveloped virus.

        An extensive selection of these viruses is

30  available, e.g., from the American Type Culture
Collection.

Targeting Ligands

        Any molecule that is capable of directing specific
interaction with a target host cell (e.g., by specific

35  recognition of and binding to a host cell surface

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protein) may be used as the targeting ligand portion of
the envelope fusion protein.  Preferably, such a protein
is derived from one member of a ligand:receptor pair.
The targeting ligands are not limited to proteins.
5  Carbohydrate and lipid moieties can be attached to the
envelope protein via protein fragments containing
consensus sequences for glycosylation and lipidation.

        Immunoglobulin genes can be used as ligands, as
shown in the example above.  Genes for high affinity
10  immunoglobulins are screened from a lambda or bacterial
expression library by a filter binding assay with
[$^{125}$I] bovive serum albumin conjugated to antigen, as
previously described (Huse, et al. *Supra*).

        Cell surface molecules such as integrins, adhesion
15  molecules or homing receptors can be used as cell-
specific ligands since they are involved in cell-cell
interactions via receptors on other cells.  Genes
encoding these molecules can be identified by the panning
method of Seed and Aruffo (Seed. B., et al., 1987, *Proc*
20  *Natl Acad Sci USA* 84:3365-3369).

        Hormones that bind to specific receptors can be
used as targeting ligands as well as viral proteins, such
as HIV envelope protein gp120, and modifications of
naturally occuring ligands.

25  <u>Therapeutic Genes</u>

        Therapeutic genes useful in the invention include
the following.   1)   Genes that are therapeutic to cancer
cells may include a) antisense oncogenes; b) tumor
suppressor genes, such as p53 or the retinoblastoma gene
30  product *Rb*, c) destructive toxin genes such as a
diphtheria toxin gene; d) cytokines such as tumor
necrosis factor or interferons; or e) any other
therapeutic gene.   2)   Therapeutic genes targeted to
cells that are infected with HIV.  Specific examples
35  include antisense DNA complementary to essential genes

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for HIV, e.g., polymerase; destructive toxin genes; e.g., diphtheria toxin; and genes that will invoke intracellular immunity, e.g., HIV enhancer sequences that titrate and remove HIV regulatory proteins (Baltimore,

5   D., 1988, *Nature* 335:395-396). 3) Genes to correct inherited deficiencies. Examples include, but are not limited to, insulin genes delivered specifically to pancreatic beta cells, or the cystic fibrosis transmembrane regulator (CFTR) gene delivered to the

10  appropriate lung cells of cystic fibrosis patients. The expression of targeted genes can be further accomplished through the use of tissue specific enhancers that regulate the transgene.

Therapy

15         For any gene therapy described herein, the appropriate recombinant virus, as described above, is administered to a patient in a pharmaceutically-acceptable buffer (e.g., physiological saline). The therapeutic preparation is administered in accordance

20  with the condition to be treated. For example, to treat an HIV-infected individual, the virus is administered by direct injection, e.g., by intravenous, intramuscular, or intraperitoneal injection, at a dosage that provides suitable targeting and lysis of HIV-infected host cells.

25  Alternatively, it may be necessary to administer the targeted virus surgically to the appropriate target tissue, or via a catheter, or a videoscope. It may be convenient to administer the therapeutic orally, nasally, or topically, e.g., as a liquid or spray. Again, an

30  appropriate dosage is an amount of therapeutic virus which effects a reduction in the disease.

        Targeted virus can also be administered by implanting viral packaging cells into a patient. The cells can be enclosed in a semi-permeable container,

35  e.g., permeable to a virus but not permeable to a

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packaging cell.  The implanted container may be
removable.  Alternatively, the container may be hooked up
to a patient intravenously, so that virus enters the
patient through a needle or through a catheter.  In this
5  way the patient receives a continuous dose of viral gene
therapy.

### Other Embodiments

Other embodiments are within the following claims.

For example, replication competent viruses may be
10 used in certain cases.  In other cases, where
replication-deficient viruses are necessary, it may be
efficacious to administer modified packaging cells,
rather than the targeted virus, to patients.  By this
method a non-proliferating dose of recombinant virus is
15 delivered to a local area, and then the virus locates the
specific target cell.  For example, tumor infiltrating
lymphocytes (TIL), which surround cancer cells, can be
modified to secrete locally high concentrations of cancer
cell-targeted virus.  Treatment may be repeated as
20 necessary.  Immune response against targeted viruses can
be overcome with immunosuppressive drugs.

In addition to colon cancer cells, the virus of
the invention may be used to target other cancer cells,
e.g., ovarian, breast, or lung cancer cells, or cells
25 affected with hereditary diseases such as muscular
dystrophy, Huntington's disease, or cells with a defect
in adenosine deaminase.  Herpesviridae viruses may
include Herpes simplex type 1 or type 2, Epstein-Barr
virus, or Cytomegalovirus.  Sendai virus and Vaccinia
30 virus may also be adapted to this method.

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## SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:             Alexander T. Young

    (ii) TITLE OF INVENTION:    GENE THERAPY USING
                               TARGETED VIRAL VECTORS

    (iii) NUMBER OF SEQUENCES:  15

    (iv) CORRESPONDENCE ADDRESS:

        (A) ADDRESSEE:      Fish & Richardson
        (B) STREET:        225 Franklin Street
        (C) CITY:          Boston
        (D) STATE:        Massachusetts
        (E) COUNTRY:      U.S.A.
        (F) ZIP:          02110-2804

    (v) COMPUTER READABLE FORM:

        (A) MEDIUM TYPE:    3.5" Diskette, 1.44 Mb
        (B) COMPUTER:      IBM PS/2 Model 50Z or 55SX
        (C) OPERATING SYSTEM: IBM P.C. DOS (Version 5.00)
        (D) SOFTWARE:      WordPerfect (Version 5.1)

    (vi) CURRENT APPLICATION DATA:

        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (vii) PRIOR APPLICATION DATA:

        (A) APPLICATION NUMBER: 07/862,795
        (B) FILING DATE:       April 3, 1992

    (viii) ATTORNEY/AGENT INFORMATION:

        (A) NAME:          Paul T. Clark
        (B) REGISTRATION NUMBER:  30,162
        (C) REFERENCE/DOCKET NUMBER:05140/002002

    (ix) TELECOMMUNICATION INFORMATION:

        (A) TELEPHONE:      (617) 542-5070
        (B) TELEFAX:       (617) 542-8906
        (C) TELEX:          200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:    1:

  (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH:              19
      (B) TYPE:                nucleic acid
      (C) STRANDEDNESS:     single
      (D) TOPOLOGY:         linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:


GCAGAAGGTC GACCCAACG                          19


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:    2:

  (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH:               10
      (B) TYPE:                nucleic acid
      (C) STRANDEDNESS:     double
      (D) TOPOLOGY:         linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:


 CCAAGCTTGG                                   10


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:    3:

  (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH:               13
      (B) TYPE:                nucleic acid
      (C) STRANDEDNESS:     single
      (D) TOPOLOGY:         linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:


GTTTTCTTTT ATC                              13


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:    4:

  (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH:               15
      (B) TYPE:                nucleic acid
      (C) STRANDEDNESS:     single
      (D) TOPOLOGY:         linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:


CAAGCATGGC TTGCC                                15


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:    5:

   (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH:                  22
        (B) TYPE:                     nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:              linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:


AGGTGCAGCT GCTCGAGTCG GG                        22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:    6:

   (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH:                  22
        (B) TYPE:                     nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:              linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:


AGGTGCAACT GCTCGAGTCT GG                        22


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:    7:

   (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH:                  22
        (B) TYPE:                     nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:              linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:


AGGTGCAGCT GCTCGAGTCT GG                        22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:    8:

   (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH:              22
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:


   AGGTGCAACT GCTCGAGTCT GG                                    22


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:    9:

   (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH:              22
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:


   AGGTCCAGCT GCTCGAGTCT GG                                    22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:    10:

   (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH:              39
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:


   CTATTAACTA GTGACGGTTA CCGTGGTCCC TTGGCCCCA                  39


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:
11:

   (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH:              45
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        double
        (D) TOPOLOGY:            linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:


AGCTTCTGCA GGCTCGAGTG ATCAACTAGT GCGGCCGCAT CGATT       45


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:   12:

   (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH:                 45
       (B) TYPE:                   nucleic acid
       (C) STRANDEDNESS:     double
       (D) TOPOLOGY:          linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:


AGACGTCCGA GCTCACTAGT TGATCACGCC GGCGTAGCTA AGATC       45


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:   13:

   (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH:                 10
       (B) TYPE:                   nucleic acid
       (C) STRANDEDNESS:     double
       (D) TOPOLOGY:          linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:


AGCGGCCGCT                                          10


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:   14:

   (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH:                 28
       (B) TYPE:                   nucleic acid
       (C) STRANDEDNESS:     single
       (D) TOPOLOGY:          linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:


CCAGCCTCCG CGGCCCCAAG CTTCTGCA              28

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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:    15:

   (i) SEQUENCE CHARACTERISTICS:

          (A) LENGTH:              24
          (B) TYPE:                nucleic acid
          (C) STRANDEDNESS:        single
          (D) TOPOLOGY:            linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:


   GGTTCTCTA GAAACTGCTG AGGGC                              24

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What is claimed is:

<u>Claims</u>

1.    A method for expressing a nucleic acid
of interest in a heterologous host cell, said method
5  comprising

(a) providing a virus whose genome comprises
(i) said nucleic acid of interest and (ii) a hybrid
envelope gene, said hybrid gene encoding an envelope
fragment joined to a targeting ligand, whereby said
10  envelope fragment does not facilitate recognition or
binding of its normal host cell but which does facilitate
efficient incorporation of said virus into a mature viral
particle and whereby said targeting ligand facilitates
targeting and binding of said mature viral particle to
15  the surface of said heterologous host cell, and

(b) administering said virus so as to permit
viral infection of said heterologous host cell.


2.    The method of claim 1, wherein said
virus is an envelope virus.


20               3.    The method of claim 2, wherein said
envelope virus is a Herpesviridae.


4.    The method of claim 2, wherein said
envelope virus is a Retroviridae.


5.    The method of claim 4, wherein said
25  Retroviridae is a Moloney murine leukemia virus.


6. The method of claim 1, wherein said
nucleic acid of interest is DNA.


7.    The method of claim 1, wherein said
nucleic acid of interest is RNA.

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8.   The method of claim 1, wherein said heterologous host cell is infectious.

9.   The method of claim 1, wherein a portion of said hybrid envelope fragment consists of a receptor
5 binding domain, an oligomerization domain, a transmembrane domain, a virus budding domain, sorting signals, and a signal sequence.

10.   The method of claim 9, wherein said envelope fragment further consists of a fusion domain.

10              11.   The method of claim 1, wherein the fusion activity of said envelope fragment is performed by a second protein.

12.   The method of claim 1, wherein said administration is by implanting a container enclosing
15 said virus into a patient.

13.   The method of claim 12, wherein said virus is inside a packaging cell.

14.   A virus, the genome of which encodes a hybrid envelope protein, said hybrid protein comprising
20 an envelope fragment joined in frame to a targeting ligand, whereby said envelope fragment does not facilitate recognition or binding of its normal host cell but which does facilitate efficient incorporation of said hybrid envelope protein into a mature viral particle and
25 whereby said non-viral protein facilitates targeting and binding of said mature viral particle to the surface of a cell not normally infected by said virus.

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15.   The virus of claim 14, wherein said virus is an envelope virus.

16.   The virus of claim 15, wherein said envelope virus is a Herpesviridae.

5        17.   The virus of claim 15, wherein said envelope virus is a Retroviridae.

18.   The virus of claim 17, wherein said Retroviridae is a Moloney murine leukemia virus.

19.   The virus of claim 14, wherein said
10   nucleic acid of interest is DNA.

20.   The virus of claim 14, wherein said nucleic acid of interest is RNA.

21.   The virus of claim 14, wherein said heterologous host cell is infectious.

15        22. The virus of claim 14, wherein a portion of said hybrid envelope protein consists of a receptor binding domain, an oligomerization domain, a transmembrane domain, a virus budding domain, sorting signals, and a signal sequence.

20        23.   The virus of claim 22, wherein said envelope fragment further consists of a fusion domain.

24.   The virus of claim 14, wherein the fusion activity of said envelope fragment is performed by a second protein.

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25. The virus of claim 14, wherein said administration is by implanting a container enclosing said virus into a patient.

26. The virus of claim 25, wherein said
5 virus is inside a packaging cell.

27. A method for delivering a nucleic acid of interest to a heterologous host cell, said method comprising
        a) providing a virus whose genome comprises
10 (i) said nucleic acid of interest and (ii) a hybrid envelope gene, said hybrid gene encoding an envelope fragment joined to a targeting ligand, whereby said envelope fragment does not facilitate recognition or binding to its normal host cell but does facilitate
15 efficient incorporation of said virus into a mature viral particle and whereby said targeting ligand facilitates targeting and binding of said mature viral particle to the surface of said heterologous host cell, and
        b) administering said virus so as to permit
20 viral infection of said cell.

28. The method of claim 27, wherein said virus is an envelope virus.

29. The method of claim 28, wherein said envelope virus is a Herpesviridae.

25                30. The method of claim 28, wherein said envelope virus is a Retroviridae.

31. The method of claim 30, wherein said Retroviridae is a Moloney murine leukemia virus.

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32. The method of claim 27, wherein said nucleic acid of interest is DNA.

33. The method of claim 27, wherein said nucleic acid of interest is an RNA.

5          34. The method of claim 27, wherein said heterologous host cell is infectious.

35. The method of claim 27, wherein a portion of said hybrid envelope fragment consists of a receptor binding domain, an oligomerization domain, a
10 transmembrane domain, a virus budding domain, sorting signals, and a signal sequence.

36. The method of claim 35, wherein said envelope fragment further consists of a fusion domain.

37. The method of claim 27, wherein said the
15 fusion activity of said envelope fragment is performed by a second protein.

38. The method of claim 27, wherein said administration is by implanting a container enclosing said virus into a patient.

20          39. The method of claim 38, wherein said virus is inside a packaging cell.

FIG. 1

FIG. 2

3/14



FIG. 3

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FIG. 4A

```
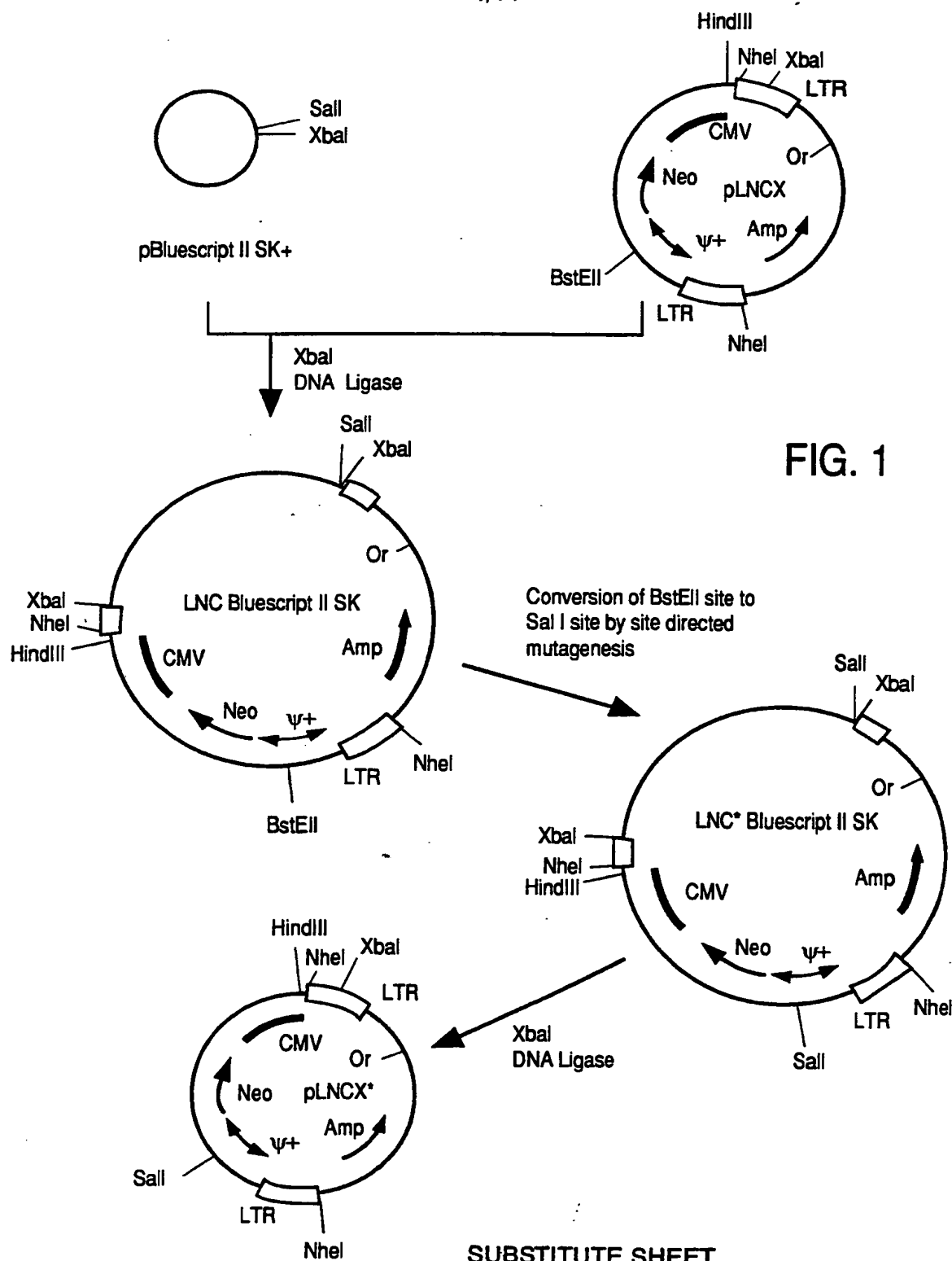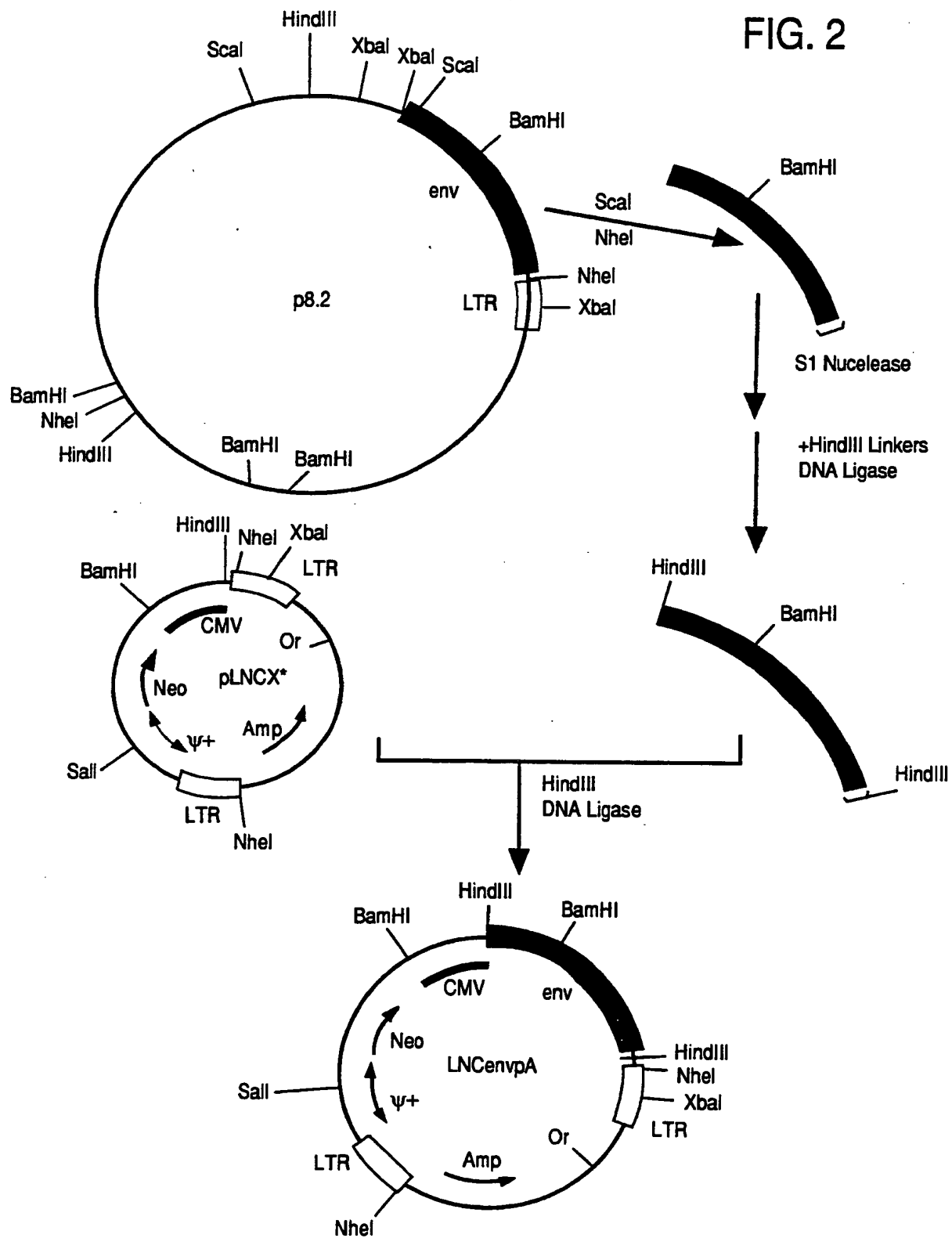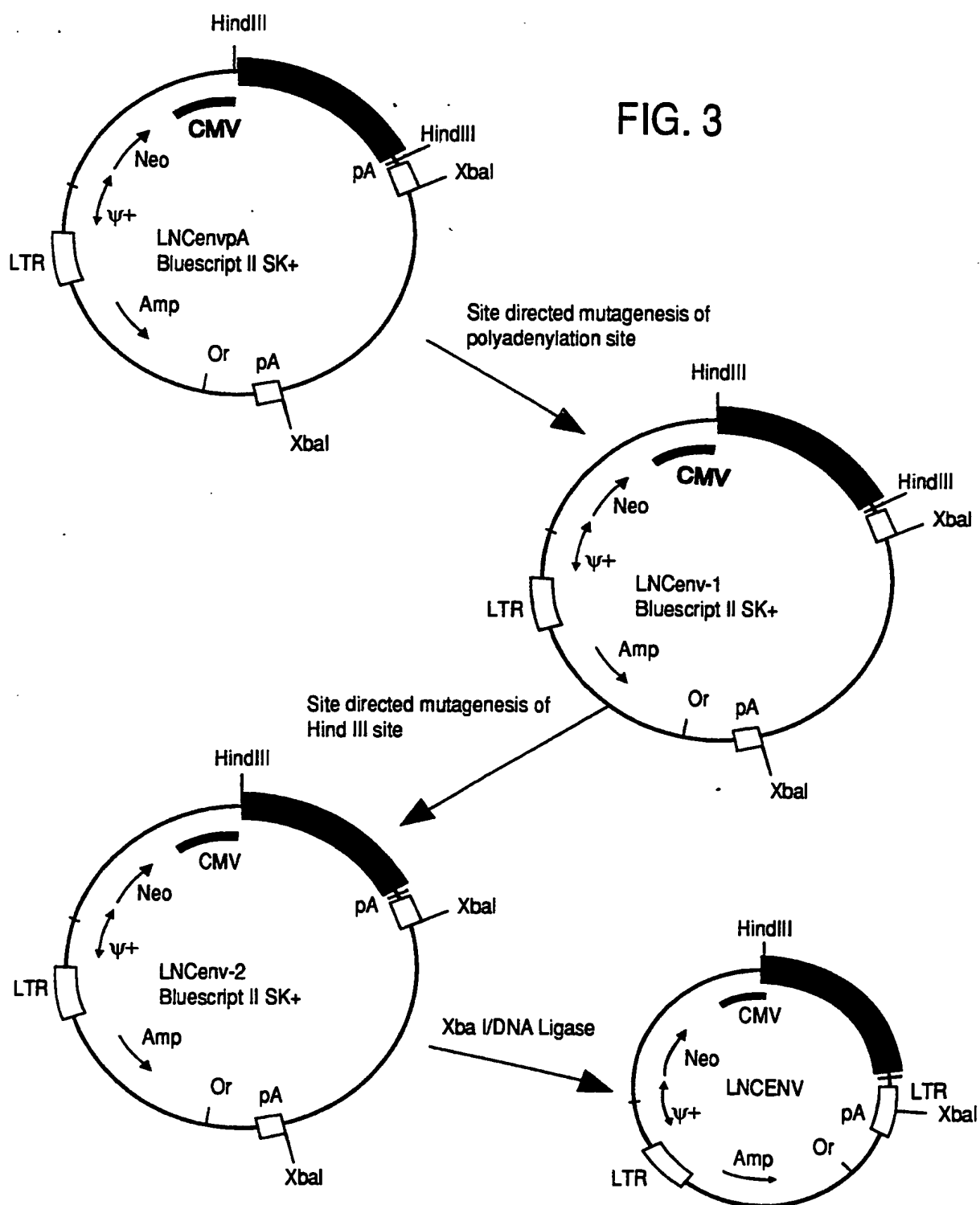                              Smal
ClaI  XbaI  BamHIXmaIKpnI  SacI  EcoRI
GCATCGATTCTAGAGGATCCCGGGTACCGAGCTCGAATTCACTGGCC
CGTAGCTAAGATCTCCTAGGGCCCATGGCTCGAGCTTAAGTGACCGG
```

Insert ~330bp Pst I
Fragment containing
immunoglobulin heavy
chain signal sequence
from clone pc DFL.1

```
HindIII PstI
ATGACCACCATGATTACGCCAAGCTTCTGCAGGGGGGGGGGGGGTTAAGGTCACT
TACTGGTGGTACTAATGCGGTTCGAAGACGTCCCCCCCCCCCCAATTCCAGTGA

GCTCTCATTACAGTTACTGAGCACACAGGACCTCACCATGGGAGCTTTCT
CGAGAGTAATGTCAATGACTCGTGTGTCCTGGAGTGGTACCCTCGAAAGA
▶MetGlyTrpSerPheLe

                                        PstI
ACCACTCTTTTGGCAGCAACAGCTACAGGTGTCCACTCCCAGGTCCAACTGCA
TGGTGAGAAAAACCGTCGTTGTCGATGTCCACAGGTGAGGGTCCAGGTTGACGT
▶uProLeuPheLeuAlaAlaThrAlaThrGlyValHisSer

                                               Smal
XhoI  BclI  Spel  NotI  ClaI  XbaI  BamHIXmaIKpnI
GGCTCGAGTGATCAACTAGTGGCGGCCGCATCGATTCTAGAGGATCCCGGGTAC
CCGAGCTCACTAGTTGATCACCGCCGGCGTAGCTAAGATCTCCTAGGGCCCATG
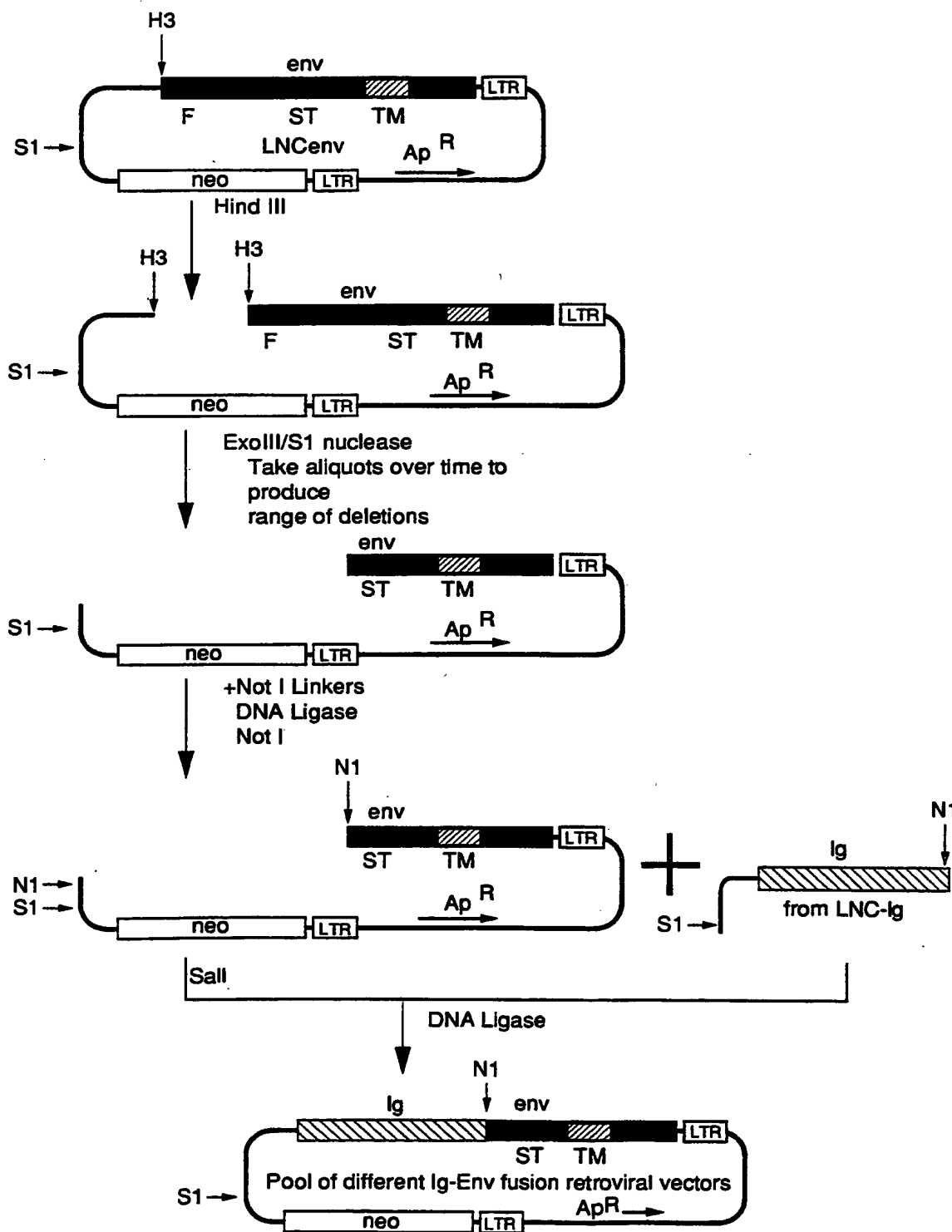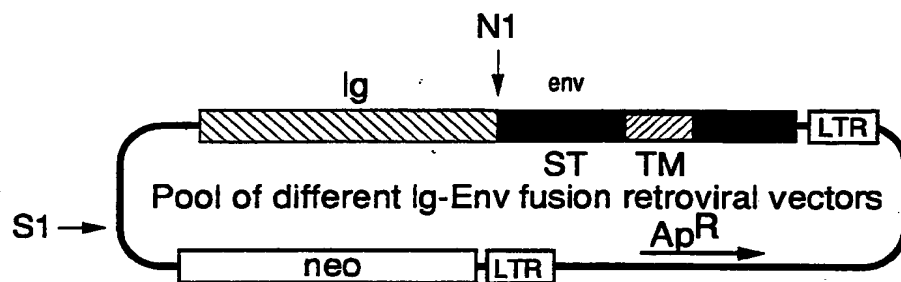
SacI  EcoRI
CGAGCTCGAATTCACTGGCC
GCTCGAGCTTAAGTGACCGG
```

pUC Star-*Sig*

FIG. 4B

HindIII   ClaI

LTR

CMV

Or

Neo

Amp

ψ+

SalI

LTR

LNCX*

ClaI
NotI
SpeI
XhoI
HindIII

pUC Star-*Sig*

Hind III/ClaI

Hind III/ClaI
Phosphatase

PstI
AGCTTCTGCAGGGGGGGGGGGGGGGGGTTAAGGTCACTGC
AGACGTCCCCCCCCCCCCCCCCAATTCCAGTGACG

TCTCATTACAGTTACTGAGCACACAGGACCTCACCATG
AGAGTAATGTCAATGACTCGTGTGTCCTGGAGTGGTAC
▶Met

GGATGGAGCTTTCTACCACTCTTTTTGGCAGCAACAGC
CCTACCTCGAAAGATGGTGAGAAAAACCGTCGTTGTCG
▶GlyTrpSerPheLeuProLeuPheLeuAlaAlaThrAl

                       PstI   **XhoI**
TACAGGTGTCCACTCCCAGGTCCAACTGCAGGCTCGAG
ATGTCCACAGGTGAGGGTCCAGGTTGACGTCCGAGCTC
▶aThrGlyValHisSer

BclI   **SpeI**   **NotI**
TGATCAACTAGTGCGGCCGCAT
ACTAGTTGATCACGCCGGCGTAGC

DNA Ligase

XhoI   SpeI   NotI
HindIII   ClaI

LTR

CMV

Or

Neo **LNC-*Sig***

Amp

ψ+

SalI

LTR

FIG. 5

FIG. 6

Bluescript SK-anti-CEA

LNC-anti CEA

FIG. 7A

FIG. 7B

300

FIG. 8

Strategy for generating targeted retroviruses
1. Construction of hybrid Ig-env genes in vitro

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FIG. 9

Strategy for generating targeted retroviruses
II. Generation of pooled virus constructions

N1

Ig   env

ST   TM

Pool of different Ig-Env fusion retroviral vectors

S1 →

neo   LTR   Ap^R

Transform ampicillin sensitive E. coll
Select Ampicillin resistant E. coii in liquid
culture
DNA prep of pool

Transfect crip 2 cells

LTR ψ neo   Ig   env   LTR

LTR Δψ   gag   pol   LTR

LTR Δψ   ENV   LTR

Harvest Virus Pools from
filtered media

Ig-env
NEO

FIG. 10   **Strategy for generating targeted retroviruses**
III. Selection and chatacterization of targeted virus

Infect Target Cells

Select G418-resistant clones
Rescue targeted virus
Sequence Ig-env gene

Retrovirus genome encoding targeing Ig-env fusion protein

FIG. 11

FIG. 12

FIG. 13

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5)   :C12N 15/86; A61K 49/00
US CL   :435/69.1, 320.1; 424/9, 93A

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/69.1, 320.1; 424/9, 93A

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG DATABASES: CA SEARCH, MEDLINE, BIOSIS PREVIEWS, WORLD PATENTS INDEX; U.S. PATENTS: APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Immunology Today, Vol. 11, No. 6, issued 1990, S. J. Russell, "Lymphokine Gene Therapy for Cancer," pages 196-200. See page 198, second column, first paragraph.	1-39
Y	Science, Vol. 250, issued 07 December 1990, J. A. T. Young et al, "Efficient Incorporation of Human CD4 Protein into Avian Leukosis Virus Particles," pages 1421-1423. See entire article.	1-39
Y	Nature, Vol. 340, issued 27 July 1989, J. W. Wills, "Retro-secretion of recombinant proteins," pages 323-324. See page 324, last paragraph.	1-39

[X] Further documents are listed in the continuation of Box C.   [ ]   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 June 1993	**18 JUN 1993**

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	JOHNNY F. RAILEY II
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

International application No.

PCT/US93/02957

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Vol. 244, issued 16 June 1989, T. Friedmann, "Progress Toward Human Gene Therapy," pages 1275-1281. See pages 1277-1278.	1-39.

Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
   (Form PCT/ISA/206 Previously Mailed.)
   I.   Claims 1, 2, 4-11, 14, 15, 17-28, 30-37, drawn to methods of expression of nucleic acids, viruses, methods of delivery of nucleic acids wherein the virus is Retroviridae.

   II.   Claims 1-3, 6-11, 14-16, 19-29 and 32-37, drawn to methods of expression of nucleic acids, viruses, methods of delivery of nucleic acids wherein the virus is Herpesviridae.

   III.   Claims 12, 13, 38 and 39, drawn to in vivo therapies for expressing nucleic acid or delivery of nucleic acids

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.
                     ☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*